Loa loa Microfilariae in Skin Snips: Consequences for Onchocerciasis Monitoring and Evaluation in *L. loa*-Endemic Areas

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The specificity of skin snips for onchocerciasis diagnoses is considered to be almost 100%. Our molecular methods revealed that microfilariae emerging from skin snips collected from highly microfilaremic *Loa loa*-infected individuals were largely misidentified as *Onchocerca volvulus*. This has important implications for onchocerciasis diagnostic testing in *Loa*-endemic areas.

Keywords. skin snip; *Onchocerca volvulus*; *Loa loa*; filariasis; diagnosis.

As the goals for the approach to onchocerciasis in Africa have shifted from morbidity control to the elimination of infection and transmission, the World Health Organization has provided new guidelines for stopping mass drug administration and verifying the elimination of human onchocerciasis [1]. These guidelines recommend parasitological evaluation by skin snip microscopy [2] to monitor progress during the first (treatment) phase of onchocerciasis elimination programs, but not to verify elimination. Indeed, it has been shown that this “gold standard” test is relatively insensitive in an early infection and when the intensity of an infection is low [3], and its usefulness in those settings near elimination and/or under surveillance has been a subject of debate [4, 5] among mathematical modelers, epidemiologists, and clinicians. Nevertheless, the consensus view of skin snip for the detection of *Onchocerca volvulus* infection is that its specificity is close to 100%. As part of a study to assess the test and not treat (TaNT) strategy for community-wide ivermectin distribution in areas hypoendemic for onchocerciasis

and co-endemic for loiasis [6], skin snips and the Standard Diagnostics (SD) Bioline Onchocerciasis immunoglobulin G4 (IgG4) rapid diagnostic test (RDT) (Ov16 IgG4 RDT) were performed in individuals excluded from ivermectin treatment because of high *Loa loa* microfilarial levels (>20 000 microfilariae [mf]/mL), to identify those coinfecting with *O. volvulus*. Surprisingly, a high percentage of seronegative individuals had positive skin snips. Given the high *L. loa* microfilarial densities in this subpopulation and prior reports of the detection of blood-dwelling filarial species in skin snips [7–9], we hypothesized that most of the mf found in the skin snips were unlikely to be *O. volvulus*, but rather *L. loa* or *Mansonella perstans*, both of which are endemic in this region. Thus, the aim of this study was to use species-specific molecular diagnostics to identify the species of mf emerging from the skin snips.

METHODS

Study Design and Patients

The present study was carried out as part of a recent study of a TaNT strategy to safely administer ivermectin for the treatment of onchocerciasis in loiasis-endemic areas. To this end, *L. loa* microfilaremia was measured in all individuals aged ≥5 years old using a device called LoaScope, and individuals harboring ≥20 000 *L. loa* mf/mL were excluded from ivermectin treatment [6]. The remainder (>97%) of the population was treated with ivermectin, without *L. loa*-related severe adverse events [6]. In order to identify excluded individuals with documented onchocerciasis for alternative treatment with doxycycline, skin snips and the Ov16 IgG4 RDT were performed.

Sample Collection and Processing

All excluded individuals were contacted in August 2017 by phone and/or in person in their respective communities, with the assistance of community leaders and local personnel trained in the framework of the TaNT strategy. Those who were found and who agreed to take part in this follow-up study underwent parasitological (LoaScope for confirmation and skin snip) and serologic (Ov16 IgG4 RDT) testing for the diagnoses of loiasis and onchocerciasis.

Confirmation of Heavy Infection With *Loa loa*

Prior to a onchocerciasis diagnosis, the LoaScope was used as previously described [6] to confirm the infection status and level of *L. loa* microfilaremia in each individual.

Skin Snips

We took 2 skin biopsies (1 from each posterior iliac crest) using a 2 mm Holth-type corneoscleral punch and, after 24

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hours incubation in saline, all emerged mf were counted under an optical microscope [10]. Positive skin snips were weighed and the microfilarial densities were expressed as the number of mf per milligram of skin (mf/mg). The saline samples containing mf were pipetted into 1.5 mL Eppendorf tubes containing isopropanol and stored at -20°C for molecular analyses.

Detection of IgG4 Antibodies Against Ov16

The Ov16 IgG4 RDT was used to identify exposure to *O. volvulus*, based on the detection of IgG4 antibodies against the *O. volvulus* Ov16 antigen [11]. The test was performed per the manufacturer's instructions, using 10 μL of finger-pricked whole blood, and was read after 20 minutes. Positive controls were performed each survey day for quality assurance purposes, prior to testing participants.

DNA Purification and Molecular Identification of Microfilariae

In May 2018, DNA was purified from mf stored in isopropanol using a DNeasy tissue Kit (QIAGEN, Valencia, CA) and a final elution volume of 200 μL . The identification of *O. volvulus*, *L. loa*, and *M. perstans* microfilarial DNA was performed using filarial species-specific quantitative polymerase chain reactions, as previously described [12, 13].

Statistical Analyses

The association between skin snip positivity and *L. loa* mf density was first explored using Cuzik's nonparametric test for trends among the quartiles of *L. loa* microfilarial densities. This association was then examined using a multilevel logistic regression model, where the dependent variable was skin snip status (positive vs negative) and the covariates were the quartiles of *L. loa* mf densities, genders, and ages. The relationship between the density of mf in skin snips and that assessed in the peripheral blood was analyzed using Spearman's correlation.

RESULTS

Parasitological and Serologic Tests for Onchocerciasis and Loiasis

A total of 173 individuals (60.8% male) aged 6 to 90 years old (median 46, interquartile range 32–60), who had been excluded from ivermectin treatment, were found and tested for the presence of skin mf using the skin snipping procedure. Of these, 172 were also tested for antibody responses using the Ov16 IgG4 RDT. Although IgG4 antibodies against *O. volvulus* Ov16 antigens were found in only 35 (20.3%) individuals tested, 134 individuals had positive skin snips (77.5%, 95% confidence interval 70.6–83.1), with microfilarial densities ranging from 0.17 to 43.94 mf/mg.

Relationship Between Infections With *Loa loa* and Skin Snip Status

The proportion of skin snip positivity tended to increase with the level of *L. loa* microfilarial density (Figure 1A, Cuzik's test $P < .001$). This was confirmed by a logistic regression, which showed that the probability of skin snip positivity was 4 to 7 times higher ($P < .005$) when the *L. loa* microfilarial density was 20 994–28 846 mf/mL, 28 847–39 178 mf/mL, and 39 179–80 900 mf/mL than when the density was below 20 993 mf/mL (reference category, Supplementary Table 1). Skin snip positivity was not associated with either age (odds ratio [OR] 0.985; $P = .167$) or gender (OR: 1.132; $P = .764$) of the study participants (Supplementary Table 1). The number of mf found in skin biopsies was positively correlated with the microfilarial density of *L. loa* in the blood (Figure 1B; rho 0.41; $P < .0001$).

Molecular Identification of Microfilariae in Skin Snips

We randomly selected 41 skin snip-positive samples, as well as 4 negative skin snip eluates, for molecular analyses. Among the 41 positive samples, molecular testing revealed that only 5 (12.2%) harbored *O. volvulus* mf. There was 1 sample that had evidence of both *O. volvulus* and *L. loa* mf, whereas only *L. loa* mf were detectable in 31 samples (75.6%). Of the 41 samples, 5

Figure 1. Relationship between *Loa loa* microfilarial density and the (A) percentage of positive skin snips and (B) numbers of microfilariae found in skin snips. Q1, Q2, Q3 and Q4 represent the quartiles of *L. loa* microfilarial densities.

(12.2%) had *M. perstans* mf. Of note, 3 and 1 of the negative skin snips contained mf from *L. loa* and *M. perstans*, respectively.

DISCUSSION

The present study aimed to identify the species of mf that emerged from skin snips, to assess the specificity of skin snips for the diagnosis of onchocerciasis in *L. loa*-endemic areas. Among individuals with positive skin snips, only 12.2% had *O. volvulus* identified by molecular assays, with the majority of skin snip positivity attributable to *L. loa* and/or *M. perstans* mf. This suggests that *L. loa* and/or *M. perstans* mf present in the dermal microcapillaries can emerge from skin snips and be misidentified as *O. volvulus*, given that the different species of mf have similar sizes, shapes, and mobility patterns on low-power microscopy. Previous studies have already demonstrated that mf of skin-dwelling *Mansonella streptocerca* [8] and blood-dwelling *Mansonella ozzardi*, *M. perstans*, and *L. loa* [7, 9] can be detected in skin snips.

This study revealed that the blood-borne parasite *L. loa* can be found in large numbers in skin snips, especially when the peripheral blood *L. loa* microfilarial density is high. Indeed, a positive association was observed between *L. loa* microfilarial densities and (1) skin snip positivity (see Figure 1A) and (2) the number of mf found in skin snips (see Figure 1B). Moreover, the majority of positive skin snips were a consequence of incorrectly identified species of the mf.

The present set of observations suggests that the presence of mf in skin snips should be used with caution to assess *O. volvulus* infection status in *L. loa* co-endemic areas, and raises important concerns related to the results of skin snip surveys for onchocerciasis that are conducted in *Loa*-endemic areas across Central Africa. Further studies might be useful (1) to assess the presence of *L. loa* in skin snips in all residents of *Loa*-endemic regions of Central Africa (not only in heavily infected individuals) and (2) to assess whether there is a *L. loa* mf density below which there is no risk of finding *L. loa* mf in skin snips. What is clear, however, is that alternative approaches to skin snipping—such as newly devised serologies that are highly sensitive and specific for *O. volvulus*—are needed to assess *O. volvulus* infections in *Loa* co-endemic regions.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the

authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. This study received ethical clearance from the Cameroon National Ethics Committee for Research in Human Health (No. 2013/11/370/L/CNERSH/SP), and an administrative authorization was granted by the Ministry of Public Health (D30- 571/L/MINSANTE/SG/DROS/CRSPE/BBM). All eligible volunteers signed an informed consent form for their participation in the study, including for the further use of their samples.

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